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Cell-mediated immunity in *Treponema pallidum* infected rabbits: *in vitro* response of splenic and lymph node lymphocytes to mitogens and specific antigens

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SUMMARY

Peripheral blood lymphocytes from *Treponema pallidum* infected rabbits respond poorly to mitogen and specific antigens when cultured in the presence of autologous serum. Reactivity of lymphocytes from the spleen and popliteal lymph nodes of *T. pallidum* infected rabbits have therefore been examined by lymphocyte transformation using the mitogens phytohaemagglutinin (PHA) and concanavalin A (Con A) and extracts of *T. pallidum*. Spleen cell populations, both T cell enriched (by nylon wool elution) and non-nylon wool treated, which respond to *T. pallidum* as early as ten days post infection in normal serum, were suppressed in responses to *T. pallidum* when cultured in autologous serum. The same lymphocytes responded normally to PHA and Con A. Lymph node cells from infected rabbits responded normally to both *T. pallidum* antigen and mitogens in either autologous or normal rabbit serum. These data indicate that splenic lymphocytes are sensitive to regulatory factors in autologous serum during the early stages of *T. pallidum* infection whereas lymph node cells are not.

INTRODUCTION

Cell-mediated immunity (CMI) in syphilis has been widely studied in the past few years, both *in vivo* and *in vitro*. Using lymphocyte transformation as an *in vitro* test of cell reactivity, several investigators have reported that peripheral blood cells from *Treponema pallidum* infected humans and laboratory animals exhibit a decreased ability to undergo blastic transformation in response to mitogens and treponemal antigens.

In rabbits, depressed CMI has been observed early in infection as evidenced by decreased *in vitro* reactivity to concanavalin A (Con A), but enhanced reactivity to Con A occurs later in infection (Pavia, Folds & Baseman, 1976). Enriched thymus-derived lymphocyte (T cell) populations obtained by nylon wool separation of peripheral blood lymphocytes (PBLs) in particular have shown depressed blastic responses to *T. pallidum* antigen and Con A when cultured in the presence of syphilitic serum (Pavia, Folds & Baseman, 1977). These findings have proved interesting, though incomplete, as only peripheral blood lymphocyte response has been studied heretofore in lymphocyte transformation. Since the spleen and lymph nodes are of primary importance as lymphoid organs, we have examined the effect of syphilitic infection on these organs in the rabbit.

Spleen cells from *T. pallidum* infected rabbits have been shown to transfer acquired cellular resistance to Listeria and delayed hypersensitivity to tuberculosis though they fail to confer resistance to *T. pallidum* (Baughn, Musher & Simmons, 1977; Schell & Musher, 1976; Schell *et al.*, 1975). This has suggested that syphilitic infection stimulates cellular immune mechanisms in the spleen against un-

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related organisms, but not against the infecting organism. We have therefore been interested in the possible effects of *T. pallidum* infection on spleen cell reactivity as measured *in vitro* by lymphocyte transformation. Since peripheral blood data suggest that the T cell response in particular may be altered and since rabbit spleens contain a large percentage of T cells, spleen cells from *T. pallidum* infected rabbits were fractionated on nylon wool columns in order to obtain a T cell enriched population for use in lymphocyte transformation assays. Also, popliteal lymph nodes were included for comparative purposes.

Lymph node cells and both unfractionated and T cell enriched populations of spleen cells were cultured in the presence of phytohaemagglutinin (PHA), Con A and *T. pallidum* antigen. The response was monitored during the first four weeks of infection by measuring uptake of ^{3}H-thymidine for the last 18 hr of culture. We report here that both unfractionated and T cell enriched populations of spleen cells exhibit a depressed blastic response to *T. pallidum* antigen in the presence of autologous serum. Lymph node cell response to the infecting organism was not suppressed by autologous serum during syphilitic infection and neither lymph node cell nor spleen cell response to the mitogens PHA and Con A was decreased during *T. pallidum* infection.

MATERIALS AND METHODS

Preparation of T. pallidum antigen. The antigen used in these studies was prepared by harvesting treponemes from cortisone-treated *T. pallidum* infected rabbits at the height of orchitis (Baseman *et al.*, 1974). The testes were aseptically removed and finely minced with scissors. Phosphate buffered saline (PBS) was added repeatedly to the minced tissue and the suspension was shaken (74 cycles/min, amplitude of 3″ stroke) at room temperature for 15 min. The fluid was removed and spun twice at 500 *g* for 5 min to remove large tissue pieces, most red blood cells, and spermatozoa. The supernatant was centrifuged over Methocel-Hypaque gradients at 650 *g* for 20 min; the top layer was removed and the treponemes were pelleted by centrifugation at 18,000 *g* for 15 min. The pellet was gently resuspended in PBS and the protein concentration determined by the method of Lowry *et al.* (1951). The suspension was then aliquoted and frozen at −20°C.

Transformation assays. Sixteen New Zealand white male rabbits were injected with 5×10^7 virulent *T. pallidum* (Nichols strain) intratesticularly. Eight control rabbits were either non-infected or injected with normal testicular extract obtained as above, but from non-cortisone treated, non-infected rabbits. Rabbits were bled for lymphocytes and autologous serum just prior to sacrifice by intravenous injection of 2 cc of Nembutal (Abbott Labs). The spleen and popliteal lymph nodes were then aseptically removed and minced by passage through 30 mesh wire screen filters (Cistron); cell debris was removed by settling. Peripheral blood lymphocytes were purified by centrifugation of whole heparinized blood at 650 *g* for 20 min over a Methocel-Hypaque gradient (Pavia *et al.*, 1977). The lymphocyte enriched bands were collected and then the cells (from spleen, lymph nodes, and peripheral blood) were washed twice in RPMI 1640 supplemented with 2 mmol glutamine (Gibco) and 100 u of penicillin/ml and 100 μg streptomycin/ml (Gibco). The peripheral blood and spleen cell suspensions were each divided into two parts, one of which was layered onto a nylon wool (Fenwal Labs) column ($6–10\times10^7$ cells/0·5 g nylon wool) (Pavia *et al.*, 1977). The columns were incubated for 30 min at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cells eluted from the column by gravity represented 90–100% T cells, as judged by dye exclusion cytotoxicity using anti-thymocyte serum (ATS) (Cappell Laboratories) and autologous rabbit complement (C′). All 5 cell populations (unfractionated spleen cells, T cell enriched spleen cells, lymph node cells, unfractionated PBLs, T cell enriched PBLs) were adjusted to a cell concentration of 3×10^6 cells/ml, 92–98% of which were judged viable by trypan blue exclusion cytotoxicity. Lymphocytes were cultured in flat-bottomed microtitre plates (Linbro) at a concentration of $4·5\times10^5$ cells/0·15 ml in RPMI 1640 plus 10% fresh pooled normal or autologous rabbit serum (heat inactivated at 56°C for 30 min). Duplicate cultures were established for each type of serum. Work was done initially using foetal calf serum (FCS) in the assays; however, since there was no difference in response of the cells when cultured in FCS than when cultured in normal rabbit serum, these studies were abandoned. Also, it has been suggested that FCS has mitogenic activity in cell culture (Coutinho & Moller, 1975). *T. pallidum* antigen and the mitogens Con A and PHA were added at concentrations previously determined by dose–response curves to give maximal stimulation (Pavia *et al.*, 1977). Cultures were incubated in a humidified chamber of 95% air and 5% CO_2 at 37°C for 48 hr. This length of time was optimal for antigen stimulation in time–response curves. Twenty μl of RPMI 1640 containing 0·5 μCi of ^{3}H-thymidine (New England Nuclear) was added to each well for an additional 18 hr incubation. Lymphocytes were harvested using a MASH cell harvester and samples were counted in a liquid scintillation spectrometer using Econofluor (NEN) as scintillation fluid.

Results are expressed as a stimulation index (SI) obtained by dividing the cpm/min of stimulated cultures by the cpm/min of unstimulated cultures. The average stimulation indices of autologous serum cultures were compared to the average stimulation indices of normal serum cultures at each time period using the Student's *t*-test for paired differences. Only significant *P* values ($P< 0·05$) are shown in Figs 1 and 2.

RESULTS

Determination of T cell percentages in the spleen

The percentages of T cells in both unfractionated spleen cell and T cell enriched preparations from *T. pallidum* infected rabbits were determined by nylon wool elution and dye exclusion cytotoxicity with ATS and C′. The percentages of T cells in peripheral blood lymphocyte populations were also determined for comparison. ATS and C′ killed between 91 and 100% of the spleen cells eluted from nylon wool columns and between 94–100% of the peripheral blood cells treated in the same manner. The percentage of T cells in the unfractionated spleen cell population dropped from 55% at day 0 of infection to 24% at 10 days post infection and remained low (36%) throughout the 30 days of infection. The percentage of T cells in peripheral blood dropped, only slightly to 33% at day 6 of infection and recovered to preinfected values of 40% by 20 days post infection.

Blastogenic responses to T. pallidum antigen

Fig. 1a–c shows the stimulation index of unfractionated and fractionated spleen cells and lymph node cells to *T. pallidum* antigen. Background values for blastogenic responses of spleen cells ranged from 566 to 5240 cpm, mean 2238±1170 for autologous serum, and from 804 to 5068 cpm, mean 2588±1086 for normal rabbit serum. Background values for lymph node cells ranged from 306 to 5763 cpm, mean 1760±920 for autologous serum and from 430 to 5761 cpm, mean 2033±995 for normal serum.

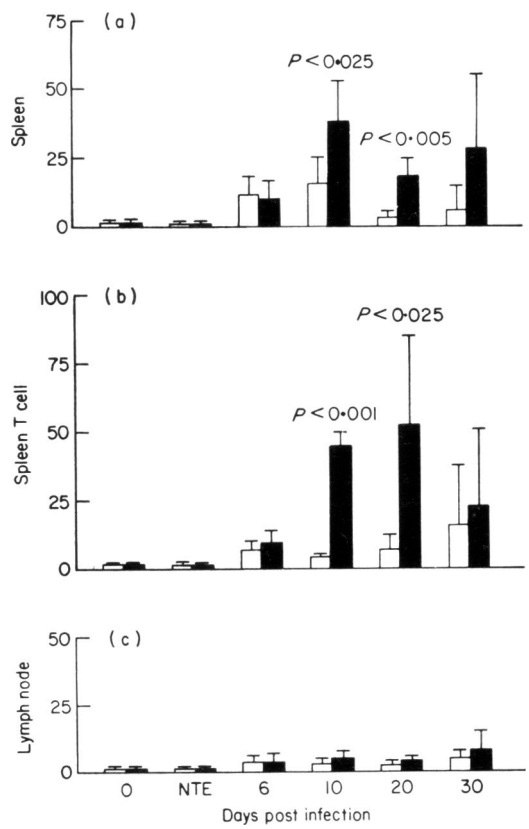

FIG. 1. (a–c) Stimulation indices (cpm of test divided by cpm of control) of (a) splenic, (b) nylon wool eluted splenic, and (c) lymph node lymphocytes from *T. pallidum* infected rabbits cultured in the presence of *T. pallidum* aud autologous (□) or normal (■) serum at various days post infection. Both uninfected control values (day zero) and normal testicular extract control values (NTE) are shown. Bars represent standard deviation. *P* values for significance as calculated by the Student's *t*-test for paired differences are also shown.

The levels of stimulation obtained with *T. pallidum* are the same in the spleen regardless of serum type only during the first six days of infection. As shown in Fig. 1a, the stimulation index (cpm of test sample divided by cpm of control) for unfractionated spleen cell populations at day 6 of infection was 12·0 in autologous serum and 10·0 in normal serum while T cell enriched cell populations (Fig. 1b) had a stimulation index of 7·0 in autologous serum and 9·5 in normal serum at that time. Beginning at day 10 of infection, lymphocytes cultured in autologous serum were markedly suppressed as compared to the lymphocytes cultured in normal serum. For instance, the stimulation index for unfractionated spleen cells at day 10 was 16·0 in autologous serum, but 40·0 in normal serum and 3·0 compared to 18·0 at day 20 post infection. Day 30 cultures had stimulation indices of 6·0 compared to 28·0; however, this difference was not significant since the normal serum cultures had a large standard deviation. The inherent variability in stimulation between outbred rabbits possibly accounts for this deviation. Stimulation values were therefore three to five-fold higher for the cells cultured in normal serum at days 10 and 20 post infection. These differences are accentuated when comparing stimulation values for T cell enriched spleen cell populations. Lymphocytes from rabbits injected with normal testicular extract as a control demonstrated the same low stimulation pattern seen in normal non-injected control animals. These data indicate that the splenic lymphocytes are responsive to *T. pallidum* antigens *in vitro* in the presence of normal serum as early as 10 days after injection with *T. pallidum*. However, some splenic lymphocytes cultured in autologous serum were unresponsive to *T. pallidum* antigens *in vitro*.

The responses of lymph nodes cells from control and infected rabbits are shown in Fig. 1c. The stimulation indices were 4·0 for both autologous and normal serum cultures at day 6 indicating only slight stimulation by *T. pallidum* at that time. As infection proceeded, these indices remained low not only in autologous serum cultures, but also in normal serum cultures and therefore did not reflect the suppression of antigenic stimulation by autologous serum seen in spleen cell cultures.

Blastogenic responses to the mitogens PHA and Con A

Fig. 2 shows the stimulation indices of unfractionated and T cell enriched spleen cell populations and lymph node cell populations to optimal concentrations of PHA and Con A. The background values for blastogenic responses in all these cell populations were described in the preceding section. As shown in Fig. 2a, the stimulation index of unfractionated spleen cells cultured in autologous serum was about the same, if not higher, than that of normal serum cultures in response to PHA at all time periods. The T cell enriched spleen cell population was also not suppressed in response to PHA in either autologous or normal serum. The profile of stimulation with Con A (Fig. 2d–f) in all cell populations is similar to that of PHA response.

DISCUSSION

Many investigators have reported suppression of peripheral blood lymphocytes during syphilitic infection, but none have monitored lymphocyte transformation in splenic or lymph node cells. Musher *et al.* (1974, 1975) examined the response of peripheral blood lymphocytes from patients with primary or secondary syphilis. They found that the peripheral blood lymphocyte response to *T. refringens* and *T. pallidum* was suppressed in patients with syphilis although the suppression was not mediated by plasma factors.

Turk and co-workers (Levene *et al.*, 1969; Friedmann & Turk, 1975) have also observed a reduction in the ability of lymphocytes from primary and secondary syphilitic patients to be transformed *in vitro*. However, a plasma factor was implicated in this suppression since plasma from secondary syphilitic patients reduced the ability of normal lymphocytes to be transformed by PHA (Levene *et al.*, 1969). Ware, Folds, & Baseman (1979), found that normal peripheral blood lymphocytes in the rabbit were suppressed with regard to blastogenic response to Con A by serum from *T. pallidum* infected rabbits.

In this investigation we studied the response of two important lymphoid organs, the spleen and lymph nodes, during *T. pallidum* infection. Splenic lymphocytes from *T. pallidum* infected rabbits were suppressed in response to specific treponemal antigens when cultured in autologous serum, but not when

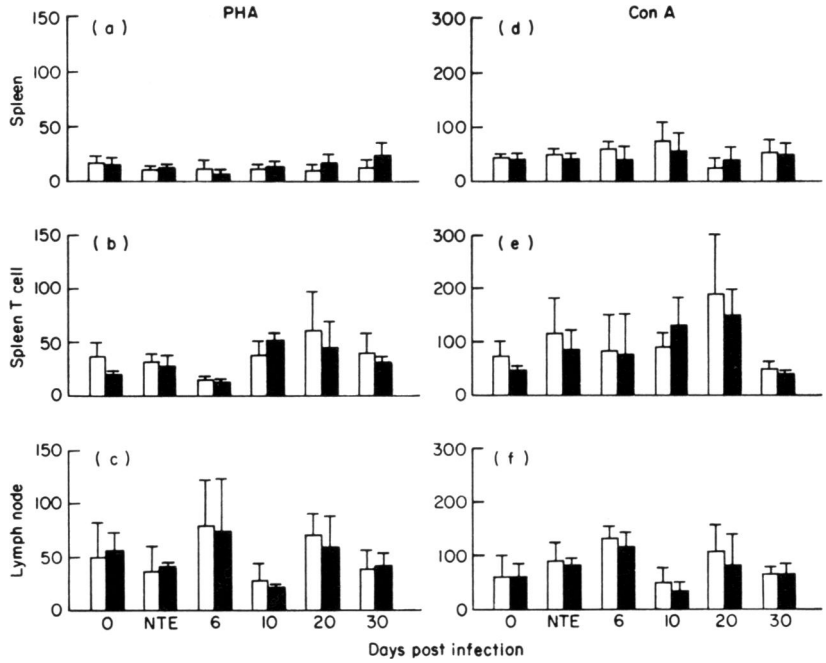

FIG. 2. (a–c) Stimulation indices of lymphocytes from *T. pallidum* infected rabbits cultured in the presence of PHA at various days post infection. (d–f) Stimulation indices of lymphocytes from *T. pallidum* infected rabbits cultured in the presence of Con A at various days post infection. Experimental conditions were as reported in the legend to Fig. 1.

cultured in normal rabbit serum. Our laboratory has reported that blastogenic response of peripheral blood lymphocytes from *T. pallidum* infected rabbits is also suppressed with respect to *T. pallidum* antigen possibly by a factor in syphilitic serum (Pavia *et al.*, 1977). Lymphocytes from the popliteal lymph nodes, however, do not exhibit depressed responsiveness in autologous serum as seen in spleen or peripheral blood.

Although peripheral blood lymphocytes were depressed in response to the mitogens PHA and Con A in the presence of syphilitic serum, both spleen and lymph node cell populations responded well to mitogenic stimulation in the presence of autologous syphilitic serum or normal rabbit serum. These data, therefore, reflect conflicts in (1) splenic and peripheral blood lymphocyte response versus lymph node cell response; (2) autologous serum response versus normal serum response among different lymphocyte populations; and (3) response to mitogen versus response to treponemal antigen. Several reasons for these discrepancies can be postulated; in particular, the presence of a factor in autologous serum which suppresses T cell activity. Also, differences in subsets of T and B lymphocytes in spleen, peripheral blood and lymph nodes as well as population dynamics may result in this differential responsiveness.

Nylon wool separation of spleen cell or peripheral blood cell populations increases the percentage of T cells in culture. Response to the T cell mitogens, PHA and Con A is therefore augmented in nylon wool separated cell populations. Response to treponemal antigen is also augmented in the T cell enriched populations, suggesting that treponemal antigens may specifically stimulate T cells. Also, passage across nylon wool appears to remove cells that modulate responsiveness to *T. pallidum*. The factor in autologous serum appears to affect specifically T cells since the T cell enriched populations are not stimulated by *T. pallidum* in cultures established in autologous serum—at least, not on the days tested in these experiments. It is also interesting that the T cell suppressive factor appears to affect only the response to *T. pallidum* antigen in spleen cells while both antigen and mitogen responses are affected in peripheral blood. It is possible that different subsets of T cells are present in the spleen, peripheral blood

and lymph nodes which give rise to the differential response to antigen and mitogen in spleen cell populations.

One must also take into account the population dynamics of the various lymphoid organs discussed here. As reported in the preceding section, there is a 50% decrease in the percentage of T cells in the spleen during the infection. Although we have not shown a concomitant increased percentage of T cells in the peripheral blood, other investigators have found that the number of T cells in peripheral blood increases almost 30% during infection (Pavia *et al.*, 1977). Reliable methods are needed for fractionating and characterizing distinct subsets of T cells (and B cells) in the rabbit before we can define the movement of lymphocyte populations during infection and the resultant modulation of the immune response in the stressed host.

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